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ULTRASTRUCTURE OF MONONUCLEAR PHAGOCYTES DEVELOPING IN LIQUID BONE MARROW CULTURES

A Study on Peroxidatic Activity*

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Kinetic studies have shown that macrophages in various tissues are derived from monocytes in the blood, which are produced in the bone marrow (1, 2). Macrophages, monocytes, and their precursor cells—promonocytes and monoblasts—can be distinguished with light microscopy (1, 3, 4), and their ultrastructural characteristics have been described in detail (5–12). These electron-microscopic studies have shown differences in the distribution of peroxidatic activity between promonocytes, monocytes, exudate and resident macrophages; exudate macrophages have the same peroxidatic activity as the blood monocytes from which they derive. The divergent distribution of peroxidatic activity in monocytes and resident macrophages has been used as evidence that the blood monocyte is not the precursor of the resident macrophages (10, 13). However, in recent studies based on the ultrastructural localization of peroxidatic activity, the transition from blood monocytes to resident macrophages was demonstrated both in vitro (14–16) and in vivo (17).

The liquid culture system offers a convenient approach to the study of proliferating bone marrow mononuclear phagocytes (4, 18, 19). The ultrastructure of these bone marrow mononuclear phagocytes in the 1st wk of culture has been described elsewhere (12).

For the present study, an attempt was made to answer the question of whether the various types of macrophages occurring in vivo (exudate, exudate-resident, and resident) also develop in liquid bone marrow cultures. Because these types of macrophages differ mainly in the localization of peroxidatic activity, special attention was given to the localization of peroxidase in mononuclear phagocytes developing in cultures.

Materials and Methods

Mouse Bone Marrow Cultures. The method used to culture bone marrow mononuclear phagocyte colonies in a liquid medium on a glass surface has been described in detail (4, 18). Bone marrow cells from specific pathogen-free Swiss mice (Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, The Netherlands) were cultured in plastic petri dishes (ϕ 60 mm; (BioQuest, BBL, & Falcon Products, Becton, Dickinson, & co., Cockeysville, Md.) Each culture contained 5×10^4 nucleated bone marrow cells per milliliter culture medium, composed of 60% (vol/vol) Dulbecco's modified Eagle's medium (Grand Island Biological

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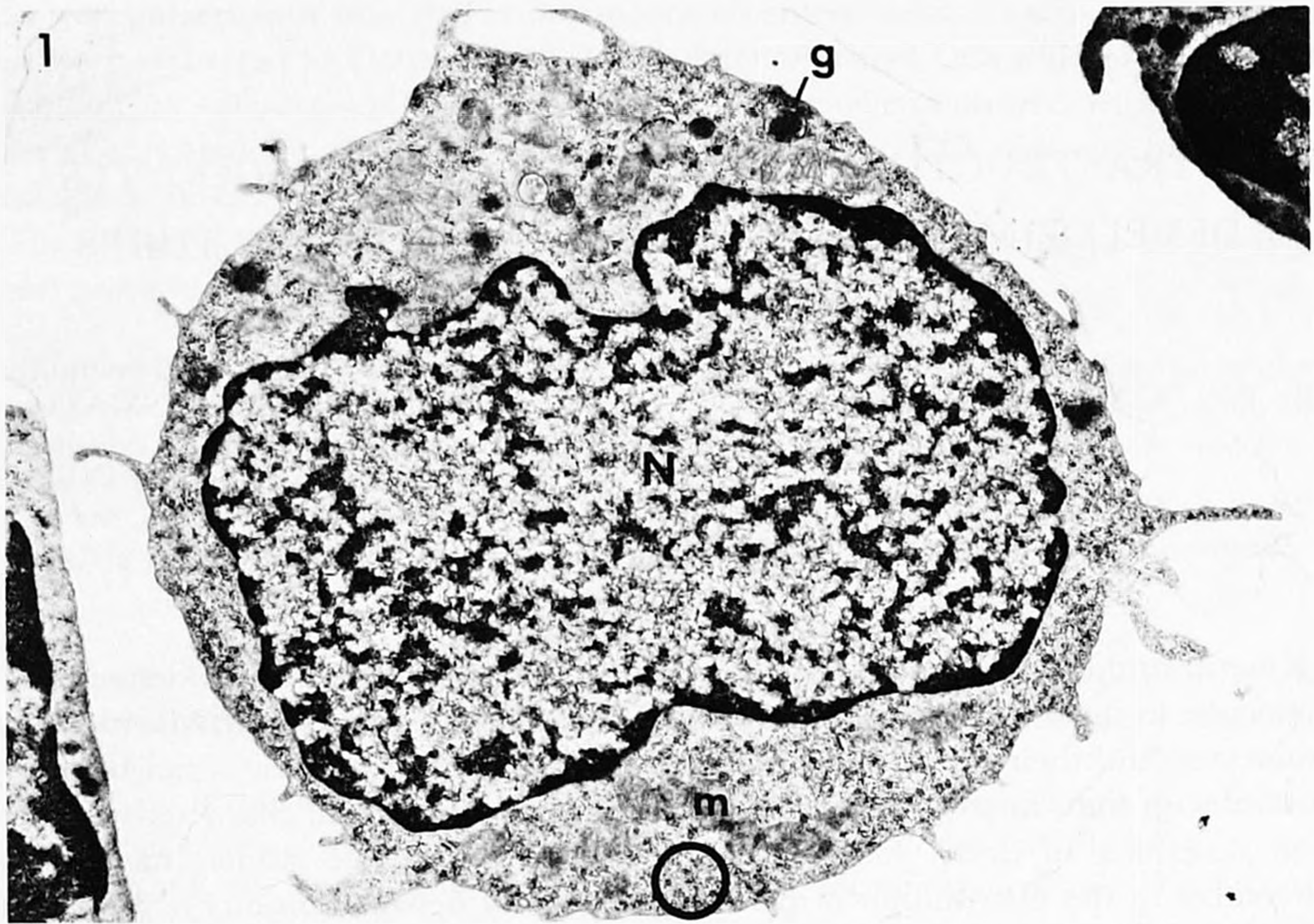


FIG. 1. Monoblast from mononuclear phagocyte colony in 2-day bone marrow culture (in the presence of conditioned medium) stained with uranyl acetate and lead citrate. The cell is round with few surface microextensions. The cytoplasm shows many polyribosomes (O), large mitochondria (m), and occasionally granules (g). The Golgi complex is not discernible. The nucleus (N) is relatively large, usually containing a nucleolus (not visible in this section). Magnification $\times 10,500$.

Company, Grand Island, N. Y.), 20% (vol/vol) horse serum (Flow Laboratories, Irvine, Scotland) and 20% (vol/vol) conditioned medium prepared from cultures of embryonic mouse fibroblasts as described elsewhere (19). The cultures were incubated in a water-saturated atmosphere with a constant flow of 10% CO_2 in air.

For the control studies, bone marrow was cultured without conditioned medium.

Ultrastructural Studies. For the morphological studies, cells were fixed for 1 h in 1.5% glutaraldehyde at 4°C , postfixed in OsO_4 for 30 min at 4°C as described earlier (17). The cells were processed in the dishes until dehydration in absolute alcohol was complete. Next, the surfaces were rinsed with propylene oxide, the detached cell layers transferred to an Eppendorf tube (Eppendorf, Hamburg, Germany) and concentrated to small pellets which were embedded in araldite (Ciba Geigy, Arnhem, the Netherlands). Ultrathin sections were cut on a ultramicrotome (Reichert, Vienna, Austria), stained with lead citrate and uranyl acetate (20) on a grid, and examined with a Philips EM 301 at 40 kV.

Cytochemistry. For the studies on peroxidatic activity, the cells were fixed for 10 min in 1.5% glutaraldehyde at 4°C , washed three times in 0.1 M Na-Cacodylate (pH 7.4), and reacted for peroxidase activity in diaminobenzidine at pH 6.5 with preincubation and incubation (0.01% H_2O_2) in the dishes for 1 h at 20°C as described in detail elsewhere (17). The cells were then washed, postfixed in OsO_4 , and further processed for electron microscopy as described above, except that the cells were examined unstained. For the control preparations, incubation was performed without H_2O_2 .

Nomenclature Used for Macrophages

RESIDENT MACROPHAGES. The term refers to macrophages with peroxidatic activity restricted to the rough endoplasmic reticulum, the nuclear envelope, and occurring in tissues and serous cavities in the normal steady state.

EXUDATE MACROPHAGES. The term refers to mononuclear phagocytes with peroxidatic

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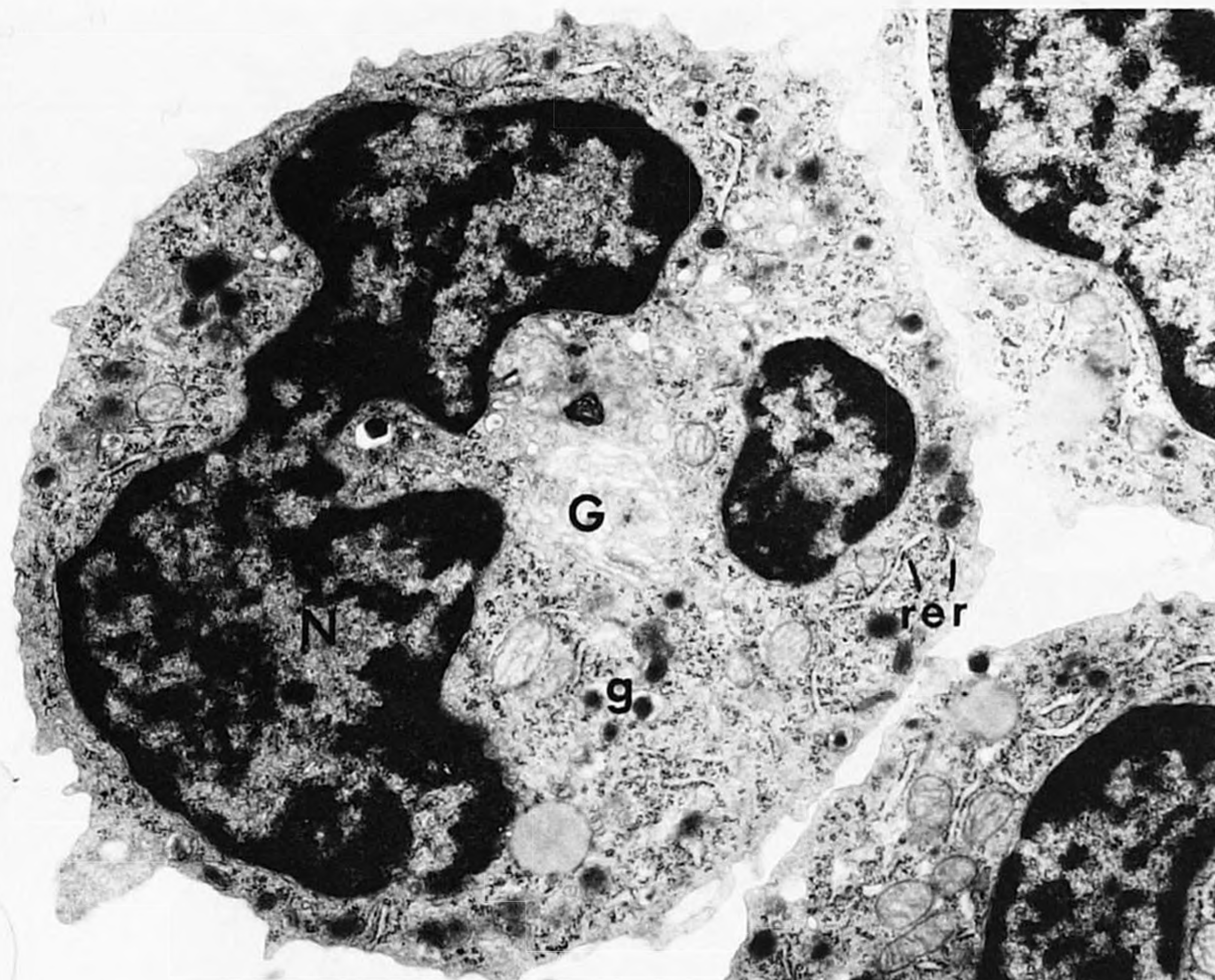


FIG. 2. Promonocyte from mononuclear phagocyte colony in 4-day bone marrow culture (in the presence of conditioned medium) stained with uranyl acetate and lead citrate. The cell shows some microextensions with occasionally a pseudopod. The cytoplasm contains less free polyribosomes than in the monoblast, however, the rough endoplasmic reticulum (rer) and the Golgi system (G) are well developed. There are a lot of granules (g). The nucleus to cytoplasm ratio is $\cong 1$. Magnification $\times 12,500$.

activity limited to the cytoplasmic granules and occurring in tissues and serous cavities during an inflammatory reaction.

EXUDATE-RESIDENT MACROPHAGES. This term is defined as mononuclear phagocytes with peroxidatic activity in granules as well as in the rough endoplasmic reticulum and the nuclear envelope and occurring in inflammatory exudate (17).

MATURE MACROPHAGES. This term refers to the mononuclear phagocytes present in cultures and showing the same distribution of peroxidase activity as the resident macrophages.

EARLY MACROPHAGES. This term refers to mononuclear phagocytes present in cultures showing the same distribution of peroxidatic activity as monocytes and exudate macrophages.

TRANSITIONAL MACROPHAGES. The term is defined as macrophages present in cultures and which show the same pattern of peroxidase activity as the exudate-resident macrophages.

Results

Long-Term Culture of Bone Marrow in the Presence of Conditioned Medium. In the presence of conditioned medium, bone marrow mononuclear phagocytes proliferate in vitro (4, 18, 19). For the recognition of these cells in the various stages of development, the characteristics described elsewhere were used (3-12, 17).

In Figs. 1 and 2 representative cells are shown. A detailed description of the ultrastructure of these various cell types is given in the legends to the figures.

As could be expected from the results of previous light-microscopic and electron-microscopic studies (4, 12, 18), the majority of cells in the first 4 days of culture consisted of monoblasts and promonocytes. Thereafter, an increasing number of cells

TABLE I
*Peroxidatic Activity of Mononuclear Phagocytes**

	Monoblast	Promonocytes	Early macro- phage	Transitional macrophage	Mature mac- rophage
Rough endoplasmic reticulum	(+)	+	—	+	+
Golgi system		(+)	—	—	—
Granules	+	+	+	+	—
Nuclear envelope	(+)	+	—	+	+

(+): Weakly positive.
* Determined by ultrastructural cytochemistry; duration of incubation not taken into account.

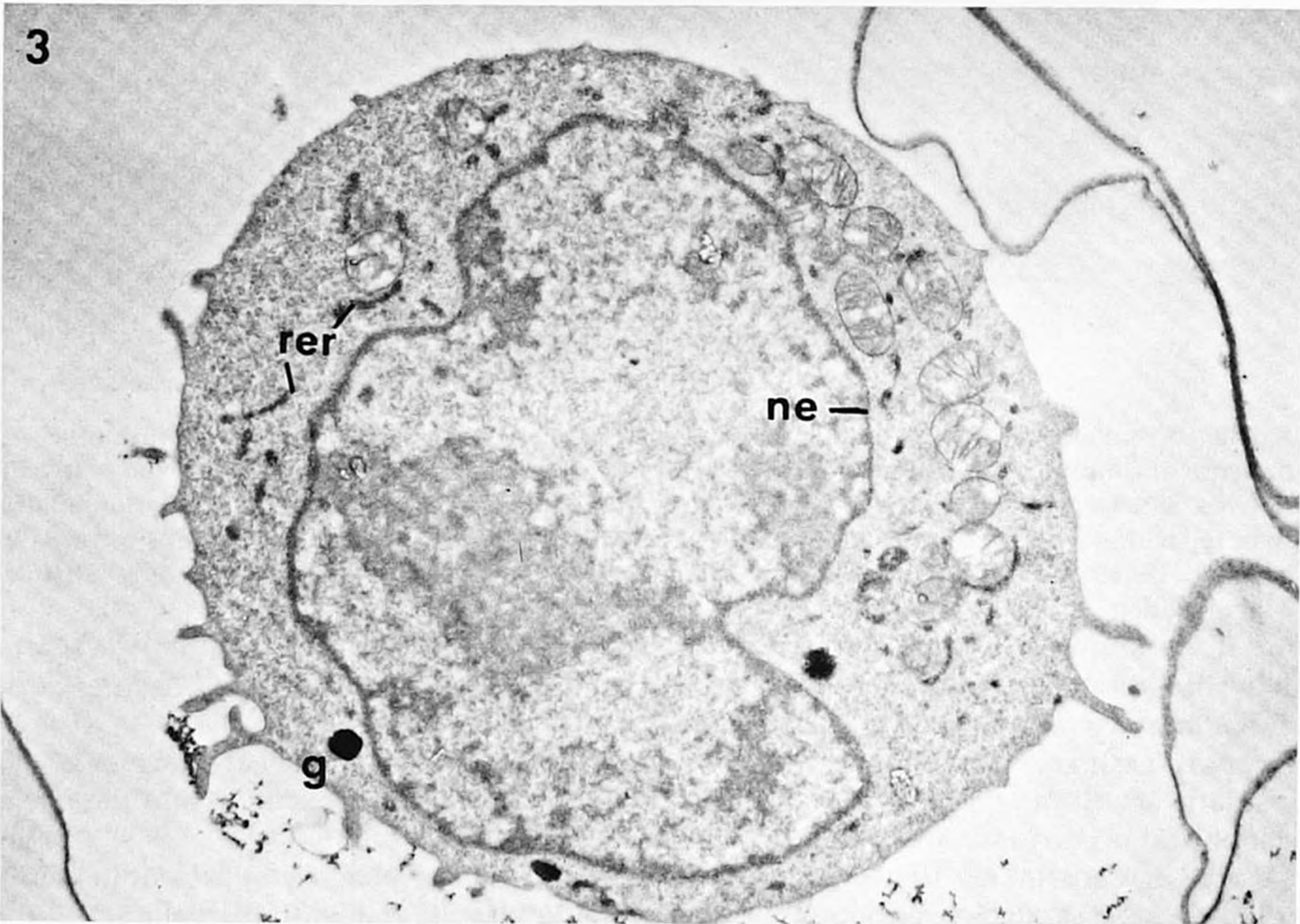


FIG. 3. Peroxidatic activity of a monoblast in a 2-day bone marrow culture (in the presence of conditioned medium). This cell shows the characteristic morphologic features of a monoblast and peroxidatic activity is visible in the nuclear envelope (ne) and rough endoplasmic reticulum (rer) and in a few large granules (g). The Golgi system is not discernible. Magnification $\times 9,000$.

with more cytoplasm, two or more pseudopods, microvilli, rough endoplasmic reticulum, and granules are seen. These macrophages cannot be differentiated further without the help of the peroxidase marker.

The peroxidase patterns observed in the various cell types are shown in Figs. 3–7 and are summarized in Table I. In the early cultures (16 h and 2 days), mainly monoblasts and promonocytes are found. Monoblasts have a few rather large peroxidase-positive granules (Fig. 3). The Golgi apparatus in the monoblast is hardly developed, if present at all, which makes it impossible to determine the peroxidatic activity of this organelle. In cultures of proliferating mononuclear phagocytes on glass or plastic, classic monocytes cannot be distinguished because the cells adhere to the surface and stretch; therefore, the cells beyond the promonocyte are referred to as macrophages.

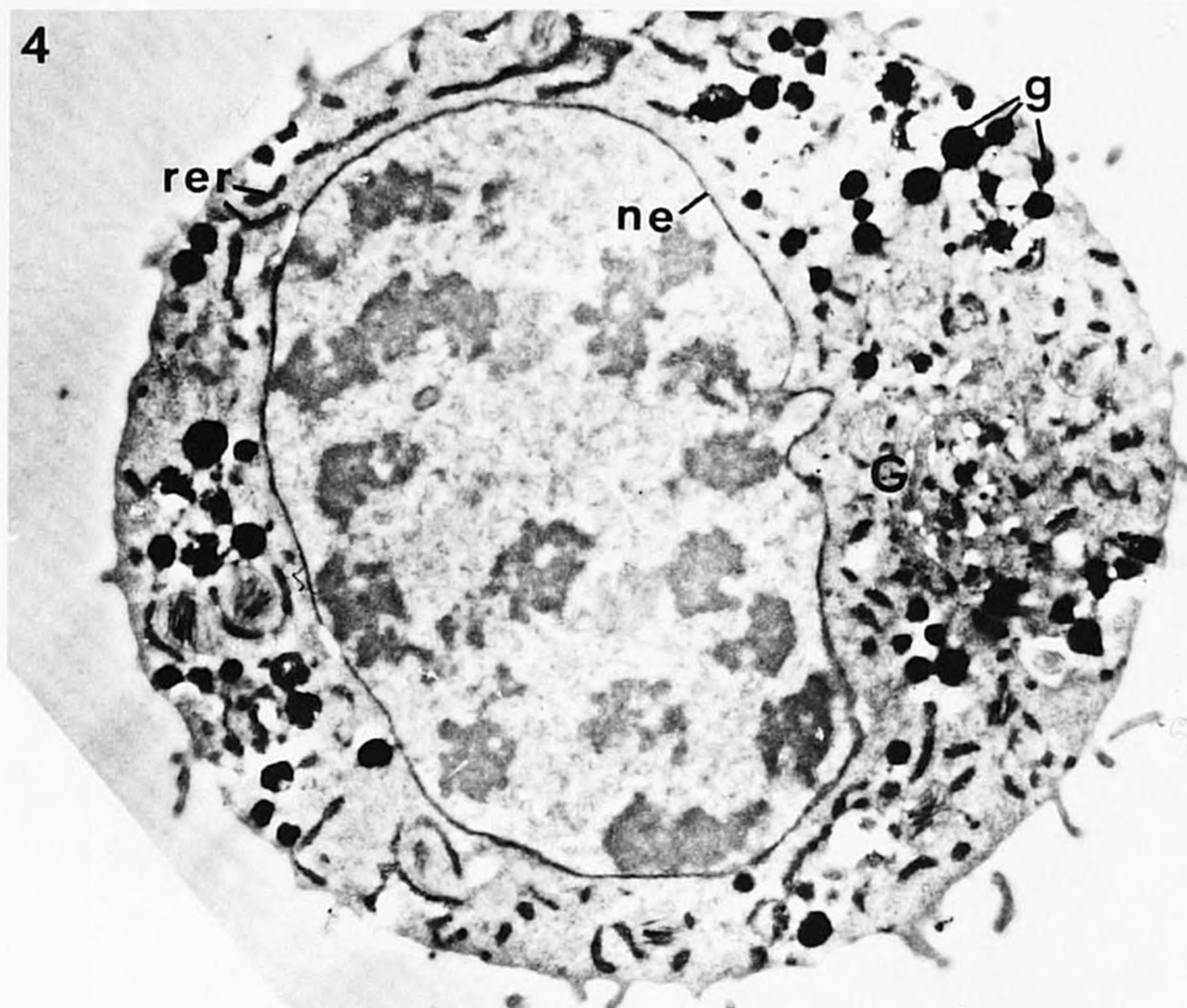


FIG. 4. Peroxidatic activity of a promonocyte in mitosis in a 4-day bone marrow culture (in the presence of conditioned medium). Peroxidatic activity is observed in the nuclear envelope (ne), the rough endoplasmic reticulum (rer), the Golgi system (G), and the granules (g). Magnification $\times 8,500$.

On the 4th day of culture, several cells with peroxidatic activity only in the granules (early macrophages) were seen (Fig. 5). A small number of macrophages with peroxidatic activity in the endoplasmic reticulum, nuclear envelope, and granules (transitional macrophages, Fig. 6), and mature macrophages with peroxidatic activity only in endoplasmic reticulum and nuclear envelope (Fig. 7), were also found. These macrophages show exactly the same pattern of peroxidatic activity as we found in resident macrophages deriving from the peritoneal cavity of the mouse. In addition, there were many monoblasts and promonocytes. The latter cells can be distinguished from the various forms of macrophages in that the Golgi apparatus has peroxidatic activity in addition to a peroxidase-positive rough endoplasmic reticulum (RER),¹ envelope, and granules; the granules in the promonocytes are larger (diameter 200–500 nm) than those in the macrophages (diameter 50–400 nm).

The relative distribution of the various developmental stages of the mononuclear phagocytes during a 16-day incubation period is given in Table II. With increasing incubation duration, the relative number of monoblasts and promonocytes decline and the various types of macrophages predominate. Early macrophages are in the majority on the 6th day of culture, after which their relative number decreases. A relatively small number of transitional macrophages are found in cultures from day 4 through day 12. However, the absolute number of these cells increases with incubation time. Mature macrophages are already present on day 4 and increase in number with the duration of incubation. On the 10th day of culture, the numbers of early, mature, and peroxidase-negative macrophages are roughly equal. Remarkably,

¹ Abbreviation used in this paper: RER, rough endoplasmic reticulum.

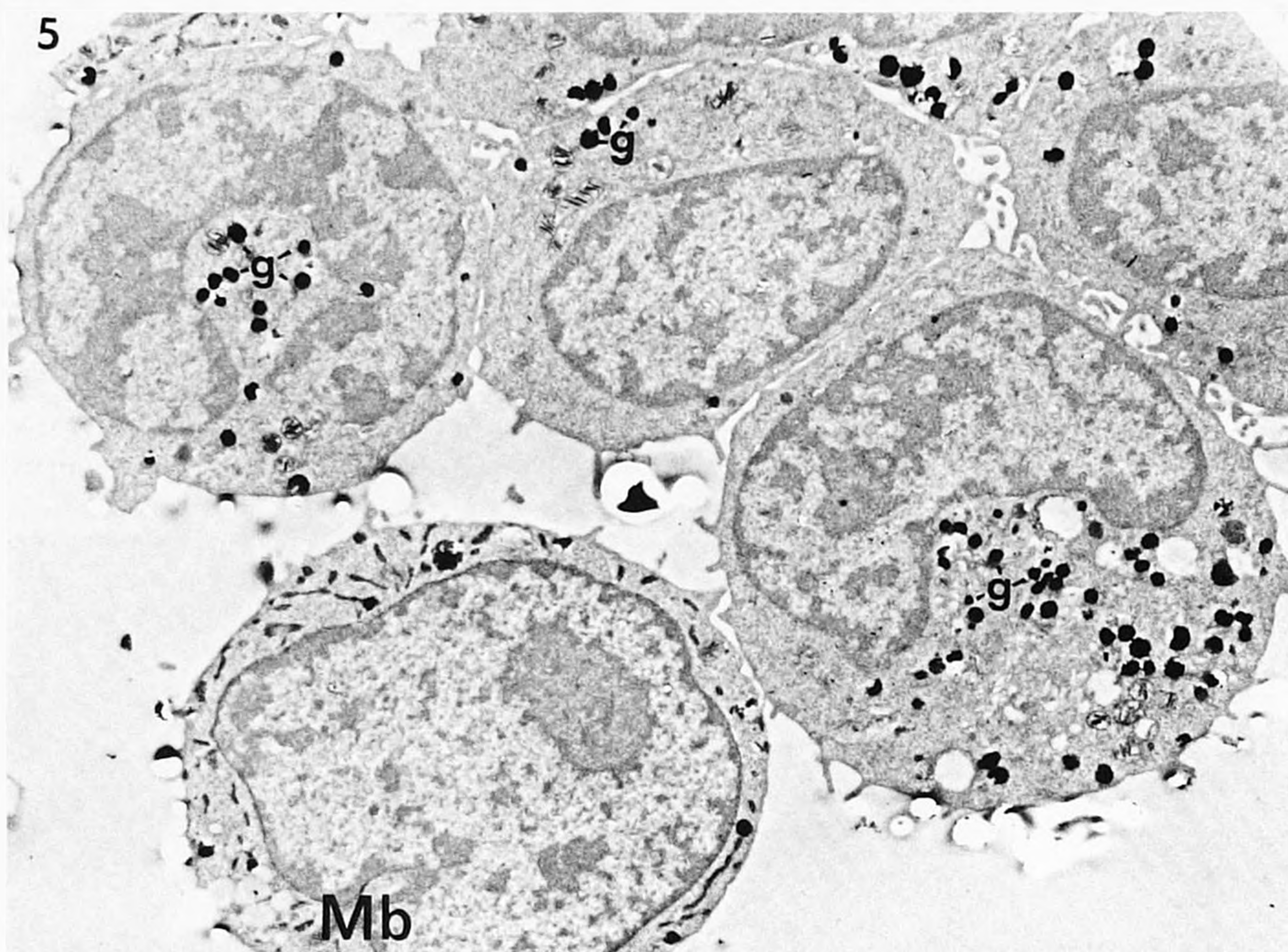


FIG. 5. Peroxidatic activity in three early macrophages of a 6-day bone marrow culture in the presence of conditioned medium. In these cells only the granules (g) are peroxidase positive. The section contains also a monoblast (Mb) with peroxidase-positive endoplasmic reticulum, nuclear envelope, and a small number of positive granules. Magnification $\times 5,000$.

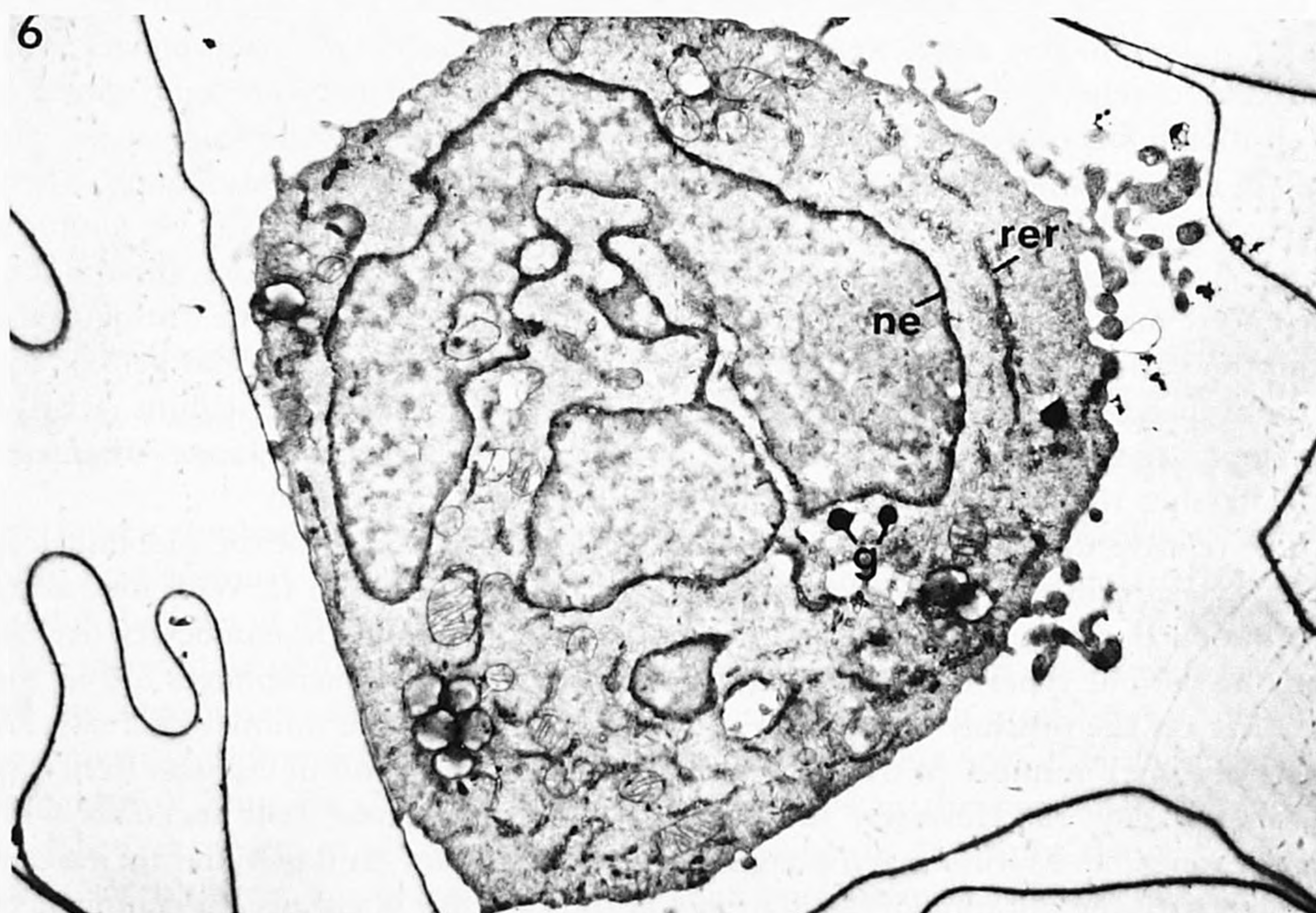


FIG. 6. Peroxidatic activity of a transitional macrophage in a 6-day bone marrow culture (in the presence of conditioned medium). The peroxidatic activity is observed in the nuclear envelope (ne), rough endoplasmic reticulum (rer), and in the granules (g). Magnification $\times 7,000$.

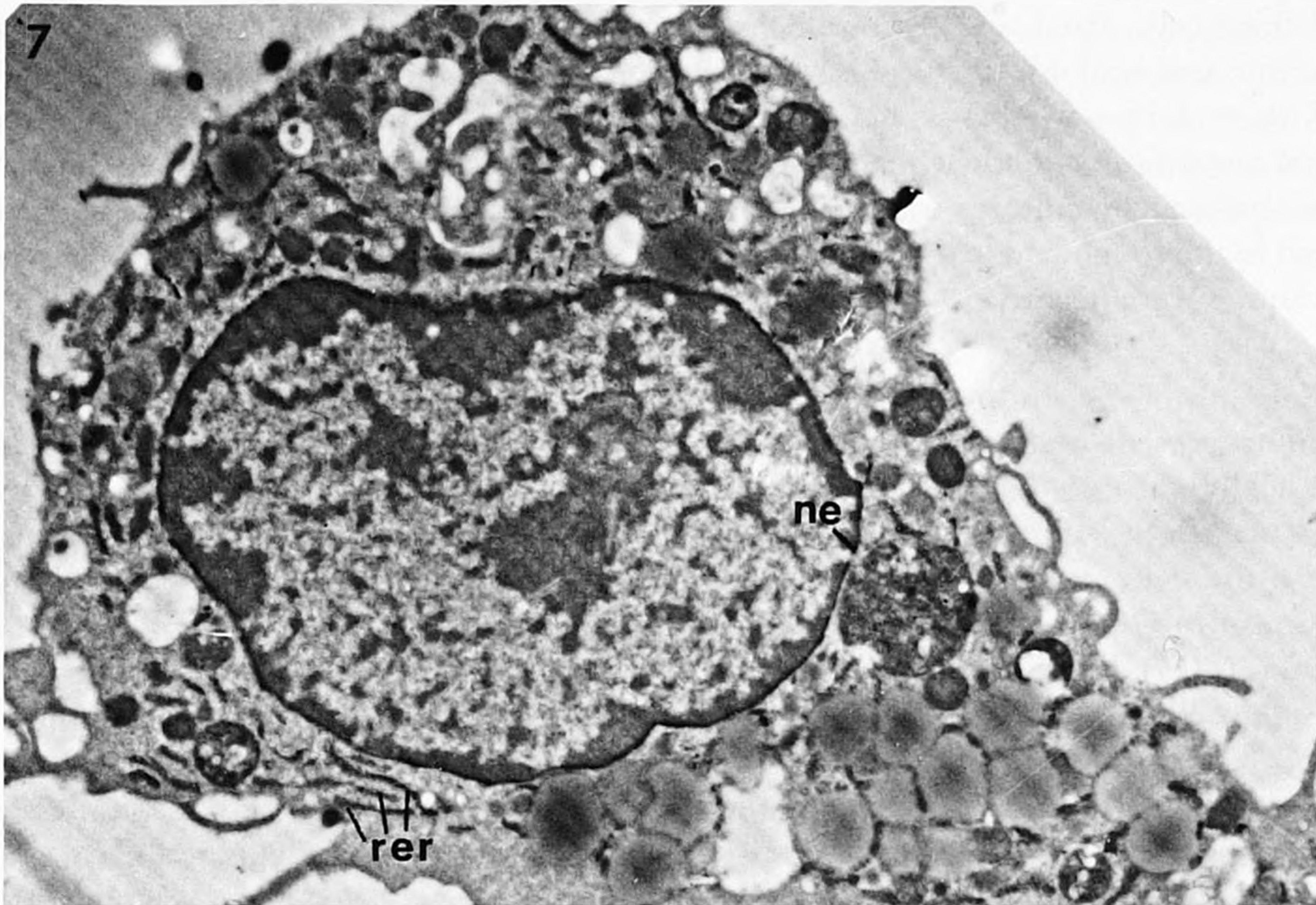


FIG. 7. Peroxidatic activity of a mature macrophage in a 14-day bone marrow culture (in the presence of conditioned medium). The peroxidatic activity is only present in the nuclear envelope (ne) and rough endoplasmic reticulum (rer). Many endocytic vacuoles are present. Magnification $\times 8,500$.

TABLE II

*Relative Distribution of Various Types of Mononuclear Phagocytes after Various Days of Incubation**

	16 h	2 days	4 days	6 days	10 days	12 days	14 days	16 days
Monoblasts	++	++	++	+	(+)	(+)	(+)	(+)
Promonocytes	++	++	++	++	(+)	(+)	(+)	(+)
Early macrophages	—	—	(+)	++	+	(+)	—	—
Transitional macrophages	—	—	(+)	(+)	(+)	(+)	—	—
Mature macrophages	—	—	(+)	(+)	+	+	+	+
Peroxidase negative macrophages	—	—	—	(+)	+	++	++	++

* On the basis of ultrastructure and of peroxidase activity. For each day of culture the relative number of cells per culture is indicated by the following symbols: (+) = a very small number of cells, + = a moderate number of cells, ++ = a large number of cells. The symbols do not indicate the course of the absolute number of cells during incubation.

the older cultures showed a considerable number of unstained cells (negative RER, Golgi, nuclear envelope, and granules).

In control preparations where H_2O_2 was omitted, occasional promonocytes were weakly reactive in the rough endoplasmic reticulum and the Golgi complex, presumably due to endogenous H_2O_2 .

Short-Term Culture of Bone Marrow in the Absence of Conditioned Medium. In the absence of conditioned medium, bone marrow cells do not proliferate, promonocytes probably divide only once, and the cells differentiate into macrophages (2, 3). The ultrastructure

of these cells, fixed with glutaraldehyde osmiumtetroxide and stained with uranyl acetate and lead citrate, has been described elsewhere (3, 7). The peroxidatic activity of these cells was reinvestigated to find out whether the macrophages acquired characteristics of resident macrophages. The results show that after 2 and 4 days of incubation, the cultures comprise $\cong 5\%$ macrophages with peroxidatic activity localized in the rough endoplasmic reticulum and the nuclear envelope, and the Golgi and granules were negative; $\cong 90\%$ of the cells did not show any peroxidatic activity.

Discussion

The present results show that the localization of peroxidatic activity changes radically during the development of mononuclear phagocytes. In bone marrow cultures, both with and without conditioned medium, mature macrophages are found with the same peroxidatic activity as seen in resident macrophages freshly harvested from mice (10, 11; R. H. J. Beelen, unpublished observations), guinea pigs (8–10), and rats (17). Immature mononuclear phagocytes, i.e., monoblasts and promonocytes, are formed in the cultures with conditioned medium during 16 incubation days; from day 4 to day 12 early macrophages, which have the same peroxidatic pattern as monocytes, were found and during the same period transitional macrophages, which have the same peroxidatic localization as exudate-resident macrophages (17), were also present. Although only relative numbers of cells are reported here, we know from quantitative studies in which light microscopy was used for the identification of cells, that during incubations; the number of monoblasts remains constant, the number of promonocytes increases more than 20-fold from day 4 to day 16, and the number of macrophages increases more than 50-fold during this period.

The present observations, showing the transition of early macrophages to mature macrophages, are not unique because *in vitro* cultures of rat, human, and rabbit monocytes (14) and rabbit and human exudate macrophages (15, 16) have also shown the development of cells with peroxidatic activity in the RER and nuclear envelope, except that none of the mononuclear phagocytes of the rabbit have peroxidase-positive granules. This pattern is similar to that of our transitional macrophage. During the course of an inflammatory reaction *in vivo*, the same changes in the peroxidase pattern are seen and a transitional cell, the exudate-resident macrophage, is found (16, 17). Although blood monocytes incubated intraperitoneally in diffusion chambers did not develop into cells with the peroxidatic pattern of either transitional macrophages or mature macrophages (13), this can probably be ascribed to the occurrence of a continuous inflammatory reaction around the diffusion chamber.

It is remarkable that in the present study a considerable number of macrophages without any peroxidase activity were found. This was also reported for human and rat mononuclear phagocytes by Bodel et al. (14) and by Beelen et al. (16, 17), but not by other authors who investigated peroxidase activity in macrophages. Analysis of murine peritoneal macrophages harvested 24 h after an intraperitoneal injection of newborn calf serum and incubated for 24 h in the absence of conditioned medium, showed that 25% of the cells have no detectable peroxidase activity (R. H. J. Beelen, unpublished observations).

The present study clearly demonstrates that transitional macrophages and mature macrophages, which have the same peroxidase pattern as exudate-resident macrophages and resident macrophages, respectively, develop *in vitro* from dividing pro-

monocytes and monoblasts. This strongly supports our view on the sequence of macrophage development in vivo, as well as in vitro: monoblast → promonocyte → monocyte → exudate macrophage (early macrophage) → exudate-resident macrophage (transitional macrophage) → resident macrophage (mature macrophage). It is conceivable that the peroxidase-negative macrophage derives from the mature macrophage; however, derivation from earlier stages is also possible.

Summary

Monoblasts, promonocytes, and macrophages in in vitro cultures of murine bone marrow were studied ultrastructurally, with special attention to peroxidatic activity. Monoblasts show peroxidatic activity in the rough endoplasmic reticulum and nuclear envelope as well as in the granules. The presence of peroxidatic activity in the Golgi apparatus could not be determined. Promonocytes have peroxidase-positive rough endoplasmic reticulum, Golgi apparatus, nuclear envelope, and granules, as previously reported.

During culture, cells are formed with peroxidatic activity similar to that of monocytes or exudate macrophages (positive granules; negative Golgi apparatus, RER, and nuclear envelope); we call these cells early macrophages. In addition, transitional macrophages with both positive granules and positive RER, nuclear envelope, negative Golgi apparatus (as in exudate-resident macrophages in vivo), and mature macrophages with peroxidatic activity only in the RER and nuclear envelope (as in resident macrophages in vivo) were found. A considerable number of cells without detectable peroxidatic activity were also encountered.

Our finding that macrophages with the peroxidatic pattern of monocytes (early macrophages), exudate-resident macrophages (transitional macrophages), and resident macrophages (mature macrophages), develop in vitro from proliferating precursor cells deriving from the bone marrow, demonstrates once again that resident macrophages in tissues originate from precursor cells in the bone marrow. Therefore, this conclusion can no longer be challenged on the basis of a cytochemical difference between monocytes and exudate macrophages on the one hand and resident macrophages on the other.

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